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The effects of chronic immune stimulation on muscle growth in rainbow trout

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Abstract

Successful production of aquaculture species depends on efficient growth with low susceptibility to disease. Therefore, selection programs have focused on rapid growth combined with disease resistance. However, chronic immune stimulation diminishes muscle growth (a syndrome referred to as cachexia), and decreases growth efficiency in production animals, including rainbow trout. In mammals, recent results show that increased levels of pro-inflammatory cytokines, such as those seen during an immune assault, specifically target myosin and MyoD and inhibit muscle growth. This suggests that increased disease resistance in fish, a desired trait for production, may actually decrease the growth of muscle, the main aquacultural commodity. To test this possibility, a rainbow trout model of cachexia was developed and characterized. A six-week study was conducted in which rainbow trout were chronically immune stimulated by repeated injections of LPS. Growth indices were monitored, and whole body and muscle proximate analyses, real-time PCR, and Western blotting were conducted to examine the resulting cachectic phenotype. Muscle ratio was decreased in fish chronically immunostimulated, however expression levels of *MyoD2* and *myosin* were not decreased compared to fish that were not immunostimulated, indicating that while muscle accretion was altered, the mechanism by which it occurred was somewhat different than that characterized in mammals. Microarray analysis was used to compare gene expression in fish that had been chronically immunostimulated versus those that had not to identify possible alternative mechanisms of cachexia in fish.

Keywords: Muscle; Cachexia; Rainbow trout; Chronic immune stimulation

1. Introduction

Selection programs focused on increasing growth rate exist for almost every economically important terrestrial species (Wheeler and Campion, 1993) and are now being developed for aquaculture species (Hulata, 2001). However, when selecting for growth, other traits are oftentimes inadvertently selected against, sometimes to the detriment of the animal's health through increased susceptibility to disease (Kocamis and Killefer, 2002; Stear et al., 2001). In selected fish, the physiological mechanisms controlling these relationships are often demonstrated as phenotypic trade-offs. For example, in both rainbow trout and hybrid striped bass, strains of fish exhibiting aggressive immune responses do not necessarily grow as efficiently as strains lacking such immune responses (Overturf et al., 2003; Li and Gatlin, 2004). Outbreaks of infectious diseases are consistently responsible for the highest percentage of losses to the trout industry on a national basis, with up to 60% of production costs being attributed to treatment of affected animals, reduced growth efficiency, and mortality losses (NASS, 2005). For this reason, there has been a great deal of effort to develop strains of rainbow trout that are disease resistant. However, because the goal of rainbow trout producers is to produce high-quality lean muscle efficiently, selection programs are faced with the challenge of balancing selection for an aggressive immune response with selection for rapid growth.

Cachexia occurs in individuals whose immune systems are chronically stimulated. The syndrome is characterized by extreme losses of adipose tissue and skeletal muscle mass, sometimes as much as 80% (Tisdale, 2002). Global effects on energy metabolism and protein turnover have often been implicated in the

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Table 1
Ingredients and proximate composition of test diet containing anchovy fish meal for rainbow trout injected with either LPS or saline

Experimental diet	
Ingredients	%
Fishmeal (anchovy) [a]	36
Corn gluten meal [a]	8
Soybean meal [a]	16
Gem Gel™ [a]	24.6
Fish oil [a]	13.0
Vitamin premix [b]	1.5
Choline chloride [a]	0.5
Vitamin C [a]	0.3
Trace mineral mix [b]	0.1
Analyzed composition [c] *(SE)*	
% Crude protein	39.1 (0.1)
% Lipid	15.1 (0.2)
Gross energy kcal/g	5784.9 (44.1)
% Ash	8.1 (0.2)
% Moisture	8.0

Diets were formulated on an as-fed basis.
Ingredients are reported as percentage of total diet on a dry matter basis. Proximate composition is reported on a dry matter basis.
[a] Origin of ingredients: Anchovy meal, corn meal, soybean meal, fish oil, and vitamin C were from Rangen, Buhl, ID, USA. Gem Gel™ and choline chloride were obtained from Nelson and Sons, Murray, UT, USA.
[b] Same as Cheng et al. (2003).
[c] Means of two replicate samples per diet.

resultant pathology (Spurlock, 1997). However, experiments using cell culture and an *in vivo* model of cachexia in mice have revealed a more specific mechanism of muscle wasting. In mouse models of cachexia, increased levels of the proinflammatory cytokines Tumor Necrosis Factor α (TNFα) and Interferon γ (IFNγ) reduced protein synthesis and stimulated protein degradation, thereby altering growth and maintenance of muscle (Tisdale, 2002; Yasumoto et al., 1995). Increased levels of TNF-α and IFN-γ inhibited the expression of *MyoD* and eventual expression of *myosin*, and also promoted the degradation of existing myosin protein via the ubiquitin-proteasome pathway (Lecker et al., 1999), resulting in fewer cells entering the myogenic pathway and decreased myosin production and stability (Acharyya et al., 2004). Together, the reduced expression of *myosin* mRNA and the increased degradation of myosin protein led to muscle wasting.

Mounting evidence indicates that chronic immune stimulation decreases growth efficiency of production animals (Spurlock, 1997). Studies in this lab and others indicate that growth efficiency is similarly decreased in chronically immunostimulated fish (Overturf et al., 2003; Bosworth et al., 1998). However, studies examining the mechanism(s) underlying chronic immune-stimulated growth suppression have not been undertaken in aquaculture species. The molecular specificity thought to underlie cachexia in mice sheds light on a possible cellular mechanism controlling the previously observed trade-offs between immune response and growth in aquaculture selection programs.

The success of commercial aquaculture depends on the ability to raise healthy fish with high quality lean muscle. Thus, the characterization and the identification of appropriate methods to enhance muscle growth in rainbow trout are necessary to increase production efficiency and profitability of this industry. If the relationships between growth and immune function in fish are similar to those observed in terrestrial animals, then it is likely that the increased immune activity exhibited by strains of fish selected for disease resistance or continuously fed diets containing immunostimulants leads to decreased muscle growth. To test the hypothesis that chronic immune stimulation alters growth efficiency of rainbow trout by suppressing muscle growth, a model of cachexia in rainbow trout was developed and characterized at the whole-body, tissue-specific, and molecular levels.

2. Materials and methods

2.1. Fish rearing and culture conditions

Fish rearing was carried out at the University of Idaho's Hagerman Fish Culture Experiment Station. Experiments were conducted in 140 L tanks with a constant temperature 15 °C spring water flow of approximately 11.5 L/min. Photoperiod was maintained at 14 h/day. All fish used were of the CSI-Oregon strain of rainbow trout. Fish were handled and treated according to the guidelines of the University of Idaho's Animal Care and Use Committee.

2.2. Time course

To develop a chronic immune stimulation model, a time course of immune response was performed using a single injection of LPS or PBS. Fish of approximately 60 g were separated into either an experimental group receiving an LPS injection or a control group receiving a sterile PBS injection. The LPS group was injected with 10 mg/kg bw (body weight) LPS in a volume of 0.1 ml sterile PBS, and the PBS group was injected with 0.1 ml sterile PBS. Liver, kidney, and muscle tissue were harvested from 10 fish in each group just prior to injection, and at 4, 8, 12, 16, 20, 24, 48, 72, and 96 h post-injection, and 7, 10, and 14 days post-injection. During the timecourse trial, fish were fed to satiation once daily with a commercial feed (Silver Cup, Murray, UT), and feed consumption was recorded daily. In the first week following injection, fish injected with LPS consumed 45–70% of the amount of feed consumed by the saline-injected fish. The following week, LPS-injected fish consumed 80–90% of the amount of feed consumed by the saline-injected fish.

2.3. Chronic immune stimulation trial

A six-week (43-day) trial of repeated LPS injections was conducted to model chronic immune stimulation. A trial duration of 6 weeks, using fish that were in a rapid growth phase, was chosen so that the effects of sustained chronic immune stimulation on muscle growth would be apparent. Fish of approximately 30 g were separated into triplicate replicate treatment groups of 55 fish each consisting of either LPS-

Table 2
Genbank accession numbers and sequences of primers and probes used for realtime PCR

Gene	Genbank accession no.	Primer/probe sequence (listed 5′–3′)
β-actin	AF254414	BactinF: CCCTCTTCCAGCCCTCCTT BactinR: AGTTGTAGGTGGTCTCGTGGATA BactinMGB: 6FAM-CCGCAAGACTCCATACCGA-NFQ
Calcyclin Binding Protein	CA042562	CacyBPF: GCAGTGGTGTCAAACTCATTCAAT CacyBPR: CCCAGGGACTGAGTTTGGAAAA CacyBPMGB: 6FAM-TCTGCACTAGGCCCTCC-NFQ
calpastatin	CA045868	CalpastatF: GGTGTCCACTTAAGAGAAAACTCACT CalpastatR: GCTGCTACGTTGCTGCAATAT CalpastatMGB: 6FAM-ATCAAGATTGGACAGACATC-NFQ
glutamate dehydrogenase	AJ419571	GludHaseF: GAGCAGAAGATGAAACGAGTGAAG GludHaseR: CTCTCCGTTGTCCCTCTTGATG GludHaseMGB: 6FAM-AAGCCCTGCAACCAC-NFQ
glutathione S transferase	AB026119	GluSTransF: GAAGAGCTTTGTCGAGAAGATGTCA GluSTransR: GGCGTCAGAGCTCAGGAA GluSTransMGB: 6FAM-CCGTCCCAAAATCA-NFQ
hepcidin	AF281354	Hep121F: TCTCCCTGTGCCGTTGGT Hep193R: GGTTCTCAGAATTTGCAGCAGAA Hep140T: 6FAM-CTGCAACTGCTGTCACAACAAGGGCT-TAMRA
hsp	CA038028	HspF: ACCAGCACCCGGTCAAAC HspR: ATCACAAGGTTTCAACGACTATCACT HspMGB: 6FAM-ATGGGAAGGAATTTC-NFQ
IL-1β	AJ004821	IL1BF: GCAGTAAAGGAGAGTACGAGTGTAG IL1BR: GCTCCATAGACCCTTCGTTGAG IL1BMGB: 6FAM-CCCAGCACTTGTTCTC-NFQ
inositol triphosphate kinase C	CA0433333	IP3KinCF: GTATGGTTTAAGTGGGAGAGGATGT IP3KinCR: TGACCTGAACCTCAATCTGACTGA IP3KinCMGB: 6FAM-ACAGGACCTCATCAAAAC-NFQ
lysozymeII	AF452171	Lys131F: TGGGTTTGCCTGTCAAAATG Lys227R: TGTTGATCTGGAAGATGCCATAGT LysT: 6FAM-TCG AGCTACAATACCCAGGCCACCA-TAMRA
TMyoD2	Z46924	MyoD-AbD-F: GCCGTCACCGACCAACT MyoD-AbD-R: CACTGTGTTCATAGCACTTGGTAGA MyoD-AbD-MGB: 6FAM-CCGTCCCATGACCCC-NFQ
myosin	Z48794	Myosin213F: TGCTGAACCTTCCTGAACTTAGA Myosin287R: GGCCTACAAGAGGCATTCTG Myosin242T: CTGGTTTGCTGCTTCCTCCGCTT-TAMRA
NFκB	CA356763	NFKBF: TGACAAAGGCATCTGCATCACA NFKBR: CATGGAGGATGCCCAGGTT NFKBMGB: 6FAM-CTGATGCTGGAGTCTTT-NFQ
prostaglandin D synthase	AF281353	PDSF: GGCTCTTGCTGGAGGATGAC PDSR: GAAGCGGCCTGGGATGT PDSMGB: 6FAM-CTGGCCAAGAAGACTG-NFQ
pyruvate dehydrogenase	CA355720	PdHaseF: GTAGTGAGGTCCCAATGTCATACTT PdHaseR: TGGGCACAGTATCTGAGTCTTCA PdHaseMGB: 6FAM-CTGCCACATCTCTCCC-NFQ
S100A1-like	CK990783	S100A1F: CATGGCACGTTCCAGTTCTG S100A1R: CCAACTCAACACTCGTGAACGA S100A1MGB: 6FAM-CCTGCAGCCATGCCGT-NFQ
SLIM1	CB512504	SLIM1F: GCATTTCTTGCAGTTGAAGCAGTA SLIM1R: GGCCATGGGACCAACGT SLIM1MGB: 6FAM-CCAGGAGTGACCCTCG-NFQ
TNFα	AJ401377	TNFF: TGGAGCCTCAGCTGGAGATATT TNFR: CCGGCAATCTGCTTCAATGTATT TNFMGB: 6FAM-CATTGGTGCAAAAGATAC-NFQ

injected or saline-injected-fish. Those groups receiving LPS treatments were injected intraperitoneally every 72 h for 43 days at a dose of 10 mg/kg bw in a volume of 0.1 ml sterile PBS. An equal volume of sterile PBS was intraperitoneally injected into control groups. Fish were fed a 40% crude protein and 15% crude lipid practical-type control diet (Hardy, 2002) that met or exceeded all known dietary requirements of rainbow trout (National Research Council, 1993) and was formulated to provide approximately half of the protein from fishmeal and half from plant sources (Table 1). Fish were fed once daily using a pair-feeding regime: initial weights of feed buckets were taken and the LPS-injected groups were fed to satiation to a maximum of 5% body weight, the amount eaten by each LPS-injected tank was determined by weighing feed buckets again, and then the

saline-injected control groups were fed the average of the amount eaten by the LPS-injected tanks. Fish were sampled on days 1, 4, 9, 15, 22, 29, 36, and 43. On each of those days, the following sampling procedure was followed: 1.) bulk tank weights were determined; 2.) three fish were removed from each tank for individual length and weight determination; 3.) the three fish used for length and weight determination were frozen at −20 °C for later whole-body proximate analysis; 4.) three separate fish were removed from each tank to determine length, weight, and fillet and liver weights; 5.) fillets used to determine fillet weights were frozen at −20 °C for later proximate analysis; 6.) liver, kidney, and muscle tissue were removed from each of the three fish that had been dissected for determination of fillet and liver weights and stored in RNA later for later RNA isolation; and 7.) muscle tissue was removed from each of the three fish that had been dissected for determination of fillet and liver weights and snap-frozen for later protein isolation. Percent weight gain, Specific Growth Rate (SGR), Condition Factor (KF), Muscle Ratio (MR), Feed Conversion Ratio (FCR), and Hepatosomatic Index (HSI) were calculated as follows: % weight gain = [(final weight (g) − initial weight (g))/initial weight (g)] * 100, SGR = [(ln final weight (g) − ln initial weight (g))/time in days] * 100, KF = weight (g)/fork length $(cm)^3$, MR = [(fillet weight (g) * 2)/whole body weight (g)] * 100, FCR = feed fed (g)/weight gain (g), HSI = [liver weight (g)/whole body weight (g)] * 100. For each sampling time point, values were determined by tank, then averaged by treatment. Student's *t*-test was performed to determine significant differences. Significance levels for all comparisons were set at $P < 0.05$.

2.4. Proximate analysis

Proximate analysis was performed to determine moisture, lipid, protein, and energy in whole-bodies and muscle fillets. Whole fish were pooled by tank at each time point and ground for homogeneity prior to analyses. Whole fish samples, individual fillet, and diet samples were analyzed in duplicate assays using standard AOAC (1995) methods for proximate composition. Dry matter and ash analysis was performed on a Leco thermogravimetric analyzer (TGA701, LECO Corporation, St. Joseph, Michigan, USA). Protein ($N \times 6.25$) was determined by the Dumas method (AOAC, 1995) on a Leco nitrogen determinator (TruSpec N, LECO Corporation, St. Joseph, Michigan, USA) and lipid was determined using a Foss Tecator Soxtec HT Solvent Extractor, Model Soxtec HT6 (Höganäs, Sweden). Total energy was determined by adiabatic bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, Illinois, USA). Protein retention and energy retention efficiencies were calculated as follows: Protein Retention Efficiency (PRE) = {[final protein (g) * final weight (g)] − [initial protein (g) * initial weight (g)]} * 100/[total feed consumed (g) * protein in feed (g)], and Energy Retention Efficiency (ERE) = {[final energy (kcal/g) * final weight (g)] − [initial energy (kcal/g) * initial weight (g)]} * 100/[total feed consumed (g) * energy in feed (kcal/g)]. For each sampling time point, values were determined by tank, then averaged by treatment. Student's *t*-test was performed to determine significant differences. Significance levels for all comparisons were set at $P < 0.05$.

2.5. RNA and protein isolation

RNA and protein were isolated from tissues at each sampling time point for later real-time PCR, Western blotting, and microarray probe synthesis. Muscle, liver, and kidney tissue collected for RNA isolation at each sampling time point was placed in RNAlater (Ambion) and stored at 4 °C for no longer than 3 weeks, according to the protocol recommended by Ambion. RNA was isolated for later real-time RT-PCR and microarray probe synthesis using the TRIzol (Invitrogen, Rockville, MD) method. Tissue was first homogenized by placing a 5 mm sterile, RNase-free, stainless steel bead into a round-bottom 2 ml tube containing TRIzol and the tissue, then placing tubes in a Retsch (Haan, Germany) MM300 Laboratory Vibration Mill for four minutes at 20 Hz. The protocol recommended by Invitrogen was followed for the rest of the RNA isolation. Tissue collected at each sampling time point for later protein isolation was snap-frozen in a dry-ice bath and stored at −80 °C until isolation. Total protein was isolated using the TRIzol method according to the protocol recommended by Invitrogen.

2.6. Quantitative real-time RT-PCR

To detect the level of gene expression at specific time points and to validate microarray results, real time quantitative RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI, according to the protocol provided by ABI (Foster City, CA). Reactions were carried out in a volume of 15 µl in 384-well plates. For *β-actin*, *CacyBP*, *calpastatin*, *glutamate dehydrogenase*, *glutathione S transferase*, *hsp*, *IL-1β*, *inositol triphosphate kinase C*, *MyoD2*, *NFκB*, *prostaglandin D synthase*, *pyruvate dehydrogenase*, *S100A1-like*, *SLIM1*, *and TNFα*, the final concentration of each reaction was: Master Mix, 1× (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference (ROX), and buffer components); MultiScribe reverse transcriptase, 0.25U/µl; RNase inhibitor mix, 0.4U/µl; forward primer, 900 nM; reverse primer, 900 nM; probe, 250 nM; total RNA, 100 ng. For *hepcidin*, *lysozyme*, and *myosin*, the final concentration of each reaction was: Master Mix, 1× (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U/µl; RNase inhibitor mix, 0.4 U/µl; forward primer, 300 nM; reverse primer, 3900 nM; probe, 200 nM; total RNA, 100 ng. For *β-actin*, *CacyBP*, *calpastatin*, *glutamate dehydrogenase*, *glutathione S transferase*, *hsp*, *IL-1β*, *inositol triphosphate kinase C*, *MyoD2*, NFêB, *prostaglandin D synthase*, *pyruvate dehydrogenase*, *S100A1-like*, *SLIM1*, *and TNFα*, primers and probe were designed by ABI's Assay by Design service. For *hepcidin*, *lysozyme*, and *myosin*, primers and probe were designed using Primer Express software (ABI). All primer and probe sequences are listed in Table 2. Cycling conditions for all genes tested except *hepcidin*, *lysozyme*, and *myosin* were as follows: 30 min at 48 °C, 10 min at 95 °C, then 40 cycles of PCR consisting

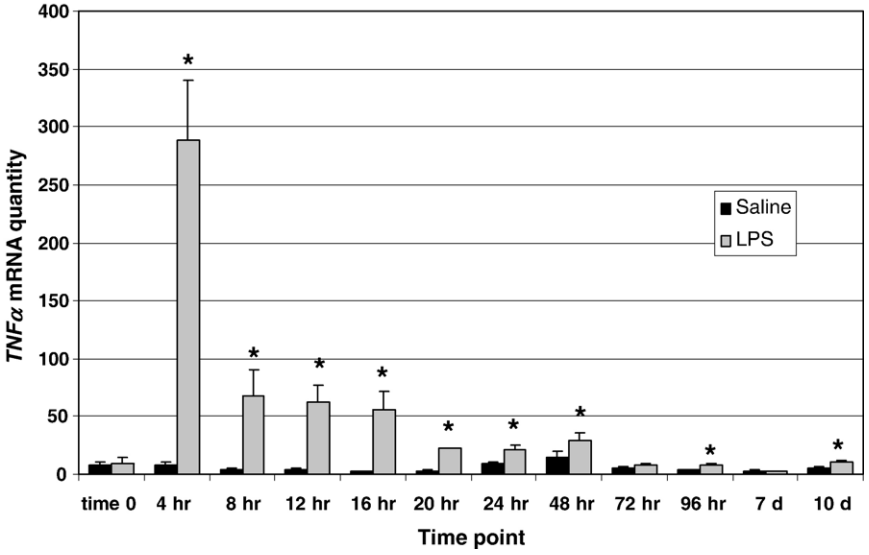

Fig. 1. Expression of *TNFα* mRNA in the kidney (relative to β-actin) after injection with either saline (black bars) or LPS (gray bars). Mean±SE is shown for each tested time point. Significant differences between saline- and LPS-injected groups at each time point are indicated by a ★ (Student's *t*-test, $P<0.05$, $n=10$).

of 15 s at 95 °C followed by 1 min at 60 °C. Cycling conditions for *hepcidin*, *lysozyme*, and *myosin* were: 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C, then 40 cycles of PCR consisting of 20 s at 95 °C followed by 1 min at 62 °C. For each group, assays were run in duplicate on RNA samples from three fish pooled by tank.

Absolute copy number of *TNFα*, *MyoD2*, and *myosin* was determined by including standards consisting of *in vitro* transcribed mRNAs specific for each gene, with each set of experimental samples that were analyzed by real-time quantitative RT-PCR. To make standards, the same primers designed for real-time amplification were used in RT-PCR of total RNA isolated from white muscle or liver tissue to amplify a fragment of each gene. Each fragment was cloned using Invitrogen's TOPO-TA cloning kit, then sequenced to determine orientation of insertion. Standards were generated by *in vitro* transcription of each clone using Promega's Riboprobe *in vitro* Transcription System (Madison, WI). Transcripts were run on formaldehyde/MOPS gels to confirm the presence of a single band of the correct size, then quantified using a spectrophotometer. The molecular weight of the *in vitro* transcribed RNA was calculated using the following formula: MW=(# of A

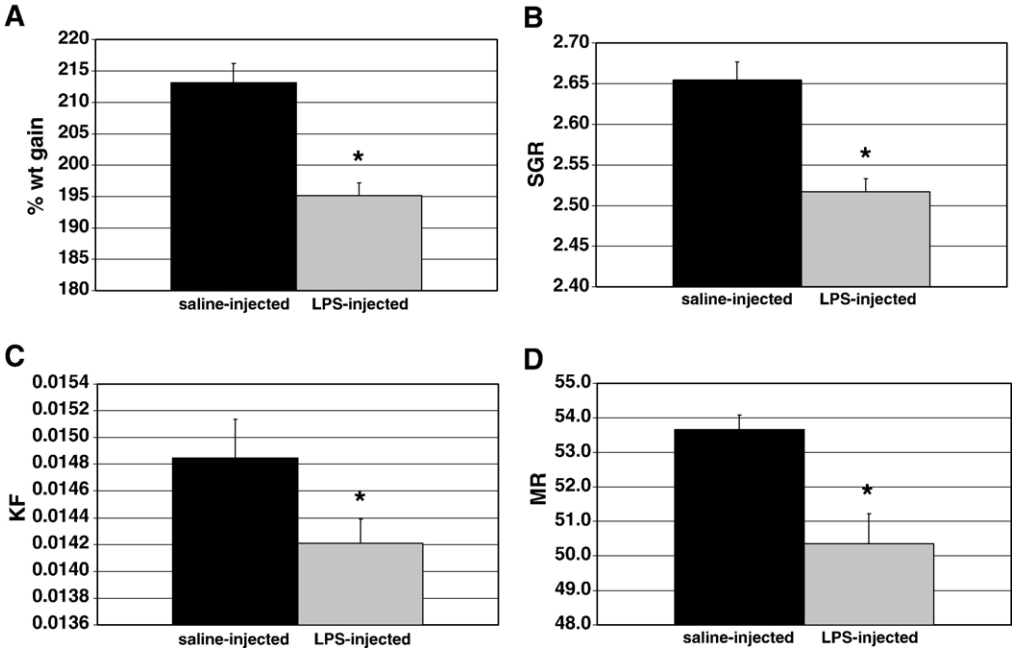

Fig. 2. Percent weight gain (A), specific growth rate (SGR) (B), condition factor (KF) (C), and muscle ratio (MR) (D) for saline-injected fish (black bars) and LPS-injected fish (gray bars). Values for % weight gain and SGR were calculated and shown for the entire 43-day study, while KF and MR are shown for day 43. Significant differences between saline- and LPS-injected groups are indicated by a ★ (Student's *t*-test, $P<0.05$; % weight gain, SGR, KF, and MR were determined for each tank, then averaged by treatment; $n=3$).

Table 3
(Muscle and whole body)[a] proximate composition of rainbow trout after 43 days of injection with (LPS or saline)[b]

Treatment	Muscle moisture	Muscle protein	Muscle lipid	Muscle energy	Whole body moisture	Whole body protein	Whole body lipid	Whole body energy	PRE[c]	ERE[d]
1 (saline)	73.15	17.57	6.79	6319	72.13	15.79	9.13	6417	45.4	155.1
2 (LPS)	74.59*	18.32	5.23*	6173	73.12	15.27	8.48	6410	40.4	186.7
P-value	0.0178	0.1793	0.0234	0.1787	0.1306	0.3080	0.0761	0.9602	0.1732	0.1767
Pooled SE	0.36	0.26	0.40	51	0.37	0.32	0.19	93	2.36	15.23

[a] Means of three replicate tanks (3 tanks/treatment) sampled at day 43.
[b] LPS values designated with a * differ significantly from control values at $P<0.05$ based on Student's *t*-test.
[c] PRE (Protein Retention Efficiency) is calculated as: [protein gain (g)*100]/protein1 fed (g).
[d] ERE (Energy Retention Efficiency) is calculated as: [energy gain (kcal/g)]*100]/energy fed (kcal/g).

bases×328.2)+(# of U bases×305.2)+(# of C bases×304.2)+(# of G bases×344.2)+159. Using the MW and concentration of each sample, copy #/μl was determined. These transcripts were used as quantitative standards to determine absolute mRNA copy number in each experimental sample. To determine expression level for all other genes, 10-fold serial dilution of a random experimental sample were prepared and included with each set of real-time runs for relative quantitation. In addition, as a cellular mRNA control, *β-actin* levels were determined for each sample and used in the normalization of specific expression data (Kreuzer et al., 1999). The data for *MyoD2*, *myosin*, and *TNFα* are reported as a ratio of absolute mRNA copy number of each specific gene to the absolute copy number of *β-actin*. Expression data was determined at each time point for each tank (RNA samples pooled from three fish per tank), and then averaged by treatment (3 tanks per treatment). Ratios were multiplied by a constant variable for ease of interpretation, and expressed as means±SE. Microsoft Excel was used to graphically represent the data. Expression data analysis was carried out using Statistica6 (Statsoft). Student's *t*-test was used for the analysis of gene expression differences. Significance levels for all comparisons were set at $P<0.05$.

Validation of microarray results (methodology reported below) was carried out using real-time RT-PCR. Data for *CacyBP*, *calpastatin*, *glutamate dehydrogenase*, *glutathione S transferase*, *hepcidin*, *hsp*, *IL-1β*, *inositol triphosphate kinase*, *lysozyme*, *prostaglandin D synthase*, *pyruvate dehydrogenase*, *S100A1-like*, and *SLIM1* are reported as a ratio of relative mRNA copy number of each specific gene to the relative copy number of *β-actin*. Expression data was determined using day 43 samples (RNA samples pooled from three fish per tank), then averaged by treatment (3 tanks per treatment). Ratios were multiplied by a constant variable for ease of interpretation, and expressed as means±SE. Microsoft Excel was used to graphically represent the data. Expression data analysis was carried out using Statistica6 (Statsoft). Student's *t*-test was used for the analysis of gene expression differences. Significance levels for all comparisons were set at $P<0.05$.

2.7. Western blotting

To detect changes in myosin protein expression in the muscles of immune-stimulated and control injected fish, Western blotting was performed. Eight μg of protein isolated from muscle of individual fish was run under reducing conditions on a 4%–20% Tris-Glycine polyacrylamide gel (Cambrex, East Rutherford, NJ) and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA). To detect myosin, the membrane was blocked for 30 min with StartingBlock solution (Pierce, Rockford, IL), and incubated in a 1:100 dilution of MF20 antibody (University of Iowa Developmental Studies Hybridoma Bank) in Antibody Dilution Buffer (200 mM Tris base, 1.37 M NaCl, 1% IgG-free and protease-free BSA, 0.05% Tween-20) overnight at 4 °C. The membrane was washed 4 times for 10 min each in Wash Buffer (200 mM Tris, 1.37 M NaCl, 0.0005% Tween-20), then incubated in a 1:5000 dilution of goat anti-mouse HRP-conjugated secondary antibody (Southern Biochemicals, Birmingham, AL) in Antibody Dilution Buffer for one hour at room temperature. The membrane was washed 4 times for 10 min each in Wash Buffer, then the West Pico chemiluminescence kit (Pierce, Rockford, IL) was used to visualize the myosin protein. Quantitation was performed by detection of pixel intensity using the Chemigenius system and GeneSnap software from Syngene.

2.8. Microarray hybridization and analysis

Microarray analysis was used to detect differences in gene expression between fish chronically immune stimulated for 43 days versus those not stimulated. Total RNA from day 43 of chronic immune stimulation was isolated by the TRIzol method from either muscle or liver and was pooled by tank (3 fish per tank), then by treatment (saline- or LPS-injected, 3 tanks per treatment), and further purified and DNase treated using Promega's SV RNA Isolation Kit. cDNA was prepared and either Cy3- or Cy5-labeled using Superscript II Reverse Transcriptase (Invitrogen) and Genisphere's Array 50 Kit. The following probe sets were made: 1. muscle RNA, saline-injected, Cy3-labeled+muscle RNA, LPS-injected, Cy5-labeled; 2. dye flip of 1.; 3. liver RNA, saline-injected, Cy3-labeled+liver RNA, LPS-injected, Cy5-labeled; and 4. dye-flip of 3. Each cDNA probe set was concentrated using Microcon YM-30 cut-off columns (Eppendorf, Hamburg, Germany), mixed with 100 μl SlideHyb 3 hybridization buffer (Ambion) and 1 μg human Cot-1 DNA (Invitrogen), incubated at 95 °C for 10 min, then held at 45 °C (hybridization temperature) until ready to use. Four slides were hybridized, one for each probe set. Hybridization was carried out using a Tecan HS400 hybridization machine. GRASP (Genomic

Research on Atlantic Salmon Project) version 1 16,008 sequence microarray slides (Rise et al., 2004) were prepared by washing for 30 s at 23 °C with 0.2 × SSC, 0.05% SDS, soaking in the same buffer for 2 min at 23 °C, and washing for 1 min at 45 °C with 4 × SSC, 0.05% SDS. Probes were injected onto slides and hybridization was carried out for 18 h at 45 °C with low agitation frequency. Slides were washed for 30 s and soaked for 1 min at 43 °C with 2 × SSC, 0.1% SDS; this step was repeated two more times. Slides were then washed for 30 s and soaked for 1 min at 25 °C with 2 × SSC; this step was repeated two more times. Slides were then washed for 30 s and soaked for 30 s at 25 °C with 0.2 × SSC, 0.05% SDS. Cy3 and Cy5 capture reagents (Genisphere, Hatfield, PA) were prepared according to Genisphere's protocol, and mixed with 50 µl Ambion SlideHyb 3 buffer and 50 µl nuclease-free H_2O. The mixture was incubated at 80 °C for 10 min, and then injected onto the slides. Hybridization was carried out for 3 h, 30 min at 45 °C with low agitation frequency. Slides were washed as described following first hybridization, and then dried for 2 min at 30 °C. Scanning was performed using a Perkin Elmer ScanArray 5000 and images and data were processed using ScanArray Express software (Perkin Elmer, Wellesley, MA). The adaptive circle method was used for quantitation, and mean background of spots was subtracted and LOWESS normalization was performed. Data was filtered by eliminating sequences that did not have a minimum of 2-fold difference in expression on at least one of the

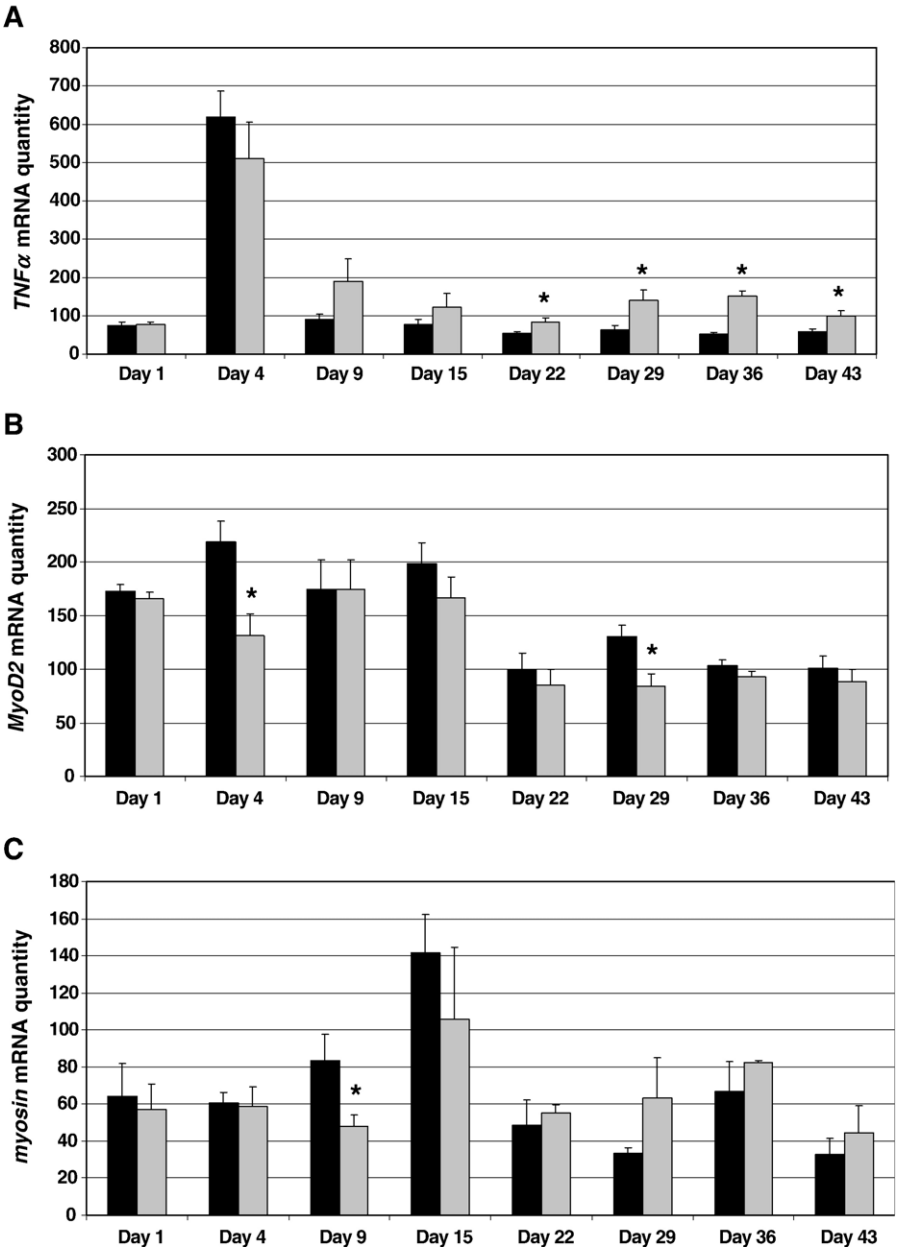

Fig. 3. Expression levels of *TNFα*, *MyoD2*, and *myosin* in the muscle of saline-injected (black bars) and LPS-injected (gray bars) fish during the chronic immune stimulation trial. Mean ± SE is shown for each tested time point. Significant differences between saline- and LPS-injected groups at each time point are indicated by a ★ (Student's *t*-test, $P < 0.05$, $n = 3$).

dye-flip slides and that did not have a spot mean minus background pixel intensity of at least 1000. Microarray results were validated by real-time RT-PCR by choosing 13 genes that were at least 2-fold different between LPS- and saline-injected groups on at least one of the dye flip slides. The 13 specific genes were selected because either primer/probe sets were already available in our lab, or because they are known to be involved in muscle growth or immune function. Real-time RT-PCR was performed using 100 ng of the same total RNA used to synthesize microarray probes, then expression differences were compared to those from the microarray analysis.

3. Results

3.1. Development of a rainbow trout model of chronic immune stimulation by timecourse analysis

A rainbow trout model of chronic immune stimulation was developed by induction of an immune response, then response phases were identified by examination of *TNFα* expression. Fish were injected with either 10 mg/kg bw lipopolysaccharide (LPS) from *E. coli* or an equal volume of sterile saline, and samples of kidney were collected and RNA isolated from five fish both prior to the initial injection and at four, eight, 12, 16, 20, 24, 48, 72, and 96 h post-injection, and at seven, 10, and 14 days post-injection. Expression level of *TNFα* was increased more than 30-fold four hours following injection of LPS (Fig. 1). Expression decreased, but remained significantly elevated through 48 h, ranging between 2- and 20-fold (Fig. 1). Two-fold elevation was observed 96 h and 10 days following injection of LPS (Fig. 1). These expression levels suggest that an acute phase response occurred four hours after immune stimulation by LPS injection, and that a chronic phase followed through 48 h, after which time a late phase ensued. Since it appeared that the chronic phase occurred through 48 h, repeated injection of LPS every 72 h was likely sufficient to maintain the immune response in the chronic phase.

3.2. Induction of cachexia by chronic immune stimulation

Fish were chronically immune-stimulated for a six-week period, then growth characteristics were analyzed using whole body indices, muscle and whole body proximate analysis, gene expression, Western blotting, and microarray analysis.

Table 4
Number of genes upregulated (U) or downregulated (D) by at least 2-fold in muscle and liver of fish injected with LPS every 72 h for 43 days

Category	Muscle		Liver	
	U	D	U	D
Immune response	13	8	15	10
Protein folding/trafficking/localization	2	4	2	3
Protein degradation	5	4	3	2
Metabolism	8	19	10	14
Transcription/translation	6	8	7	9
Cell cycle/cell death	5	6	2	5
Growth/development	7	7	3	10
ECM/cytoskeleton	5	1	3	1
Unidentified	51	71	93	67
Total	101	128	138	121

Table 5
Real-time PCR validation of array results

Gene name	Array[a]	Real-time PCR[a]
Calcyclin Binding Protein	+6.56	+1.15
calpastatin	+1.70	+1.89
SLIM1	−1.83	−1.76
IP3KC	−191.3	−1.35
S100A1-like	+1.40	−1.40
IL-1β	+1.73	+1.32
lysozyme	+3.67	+4.32
hepcidin	−4.10	+3.08
pyruvate dehydrogenase	+1.72	+1.51
glutamate dehydrogenase	−1.73	−1.01
hsp	+1.78	−1.02
lysozyme (L)[b]	+2.72	+2.35
prostaglandin D synthase (L)[b]	−1.77	−1.86
glutathione S transferase (L)[b]	−1.65	−1.31

[a] Values represent the fold difference in expression of genes from tissues of LPS-injected fish compared to saline-injected detected by the microarray and by realtime PCR.
[b] With the exception of gene names followed by (L), validation was done using RNA from muscle.

3.2.1. Whole-body indices

Over the course of the six week study, there were significant differences in weight gain and specific growth rate, with LPS-injected fish gaining less weight and exhibiting lower specific growth rates than their saline-injected counterparts (Fig. 2A, B). By day 43 of chronic immune stimulation, condition factor (KF) was lower in immune stimulated fish (Fig. 2C) and muscle ratio was significantly lower in the LPS-injected group than in the saline-injected group (Fig. 2D). Feed conversion ratio (FCR) and hepatosomatic index (HSI) did not change with LPS treatment (data not shown).

3.2.2. Proximate analysis

By day 43 of the chronic immune stimulation trial, muscle fillet moisture was significantly increased in LPS-injected fish, while lipid was significantly decreased (Table 3). Neither muscle protein nor energy were changed, nor were whole body moisture, protein, lipid or energy (Table 3). Protein retention efficiency and energy retention efficiency were also not significantly different between the two treatments (Table 3).

3.2.3. Gene expression

At most time points, expression of *TNFα* was higher in fish injected with LPS (significantly higher at days 22, 29, 36, and 43) (Fig. 3A), indicating that immune stimulation was achieved. To test whether chronic immune stimulation inhibits muscle growth in fish in a manner similar to that in mammals, i.e., specific inhibition of both *MyoD* and *myosin* expression, real-time PCR was used to test the expression of these factors in muscle samples taken throughout the study. In LPS-injected groups, *MyoD2* expression was significantly lower at days four and 29 (Fig. 3B), and *myosin* expression was significantly lower at day 9 (Fig. 3C), of chronic immune stimulation. Neither *MyoD2* nor *myosin* showed

consistent reduction in expression as has been observed in mammals, suggesting that the mechanism of immune-stimulated cachexia in fish may be different than that observed for mammals.

3.2.4. Western blotting

Acharyya et al. (2004) demonstrated that cachexia occurs though molecular inhibition of myosin at both the RNA and protein level. To determine whether chronic immune stimulation in fish causes a decrease in myosin protein, Western blotting was performed on protein samples isolated from fish that had undergone 43 days of immune stimulation by LPS injections or 43 days of control saline injections. The mouse monoclonal MF-20 antibody, raised against chicken pectoralis muscle myosin heavy chain and demonstrated to react with rainbow trout myosin (Biga et al., 2004), detected no consistent differences in myosin protein level between chronically immune-stimulated fish and their control counterparts (data not shown), lending additional support for an alternative cachectic mechanism in fish.

3.2.5. Microarray analysis

Since the mechanism of immune-stimulated muscle wasting appeared to be different in fish than in mammals, a microarray hybridization experiment was performed to determine differences in gene expression between LPS- and saline-injected groups. RNA samples from both the muscle and liver of LPS-injected groups and saline-injected groups at day 43 were reverse transcribed, labeled, and used to probe the GRASP 16,008 sequence salmonid microarray chip (Rise et al., 2004). One set of hybridizations was performed to examine the differences in gene expression in the muscle of LPS- vs. saline-injected fish, and another set was performed to examine the differences in gene expression in the liver of LPS- vs. saline-injected fish. For each tissue, expression levels of over 200 genes were at least two fold different between LPS- and saline-injected groups (Table 4). Complete data tables listing expression changes for all sequences on the array have been deposited on the NCBI Gene Expression Omnibus (GEO) with the accession number of GSE4790.

Microarray results were validated by testing expression by real-time PCR of 13 genes that were at least 2-fold different between LPS- and saline-injected groups on at least one of the dye flip slides. Eleven of the 13 genes tested showed expression differences detected by real-time PCR to be up- or downregulated similarly to differences detected by the microarray (Table 5). The most dissimilar expression difference between the array and real-time PCR was shown for *hepcidin*, and may have been due to real-time PCR probe and primer sequences that are slightly different than the sequence of the EST spotted on the array.

In both liver and muscle tissue from chronically immune-stimulated fish, the expression of several genes involved in immune response was affected (Table 4). For example, sequences encoding lysozyme and Ig chains were upregulated in both liver and muscle. Additionally in liver, expression of sequences encoding proteins involved in inflammation, such as TNFα and prostaglandin D synthase, were altered. Expression of several genes considered to be part of the acute phase response (reviewed by Bayne and Gerwick, 2001) were altered with LPS treatment. For example, genes encoding α2-macroglobulin in the muscle, haptoglobin in both the muscle and liver, and a precerebellin-like protein and C-type mannose-binding lectin in the liver, were upregulated.

A number of metabolism-related genes, specifically genes involved in the transport of cholesterol and the mobilization of fatty acids, were altered with chronic immune stimulation. In the liver, genes encoding StAR (Steroid Acute Regulatory protein), a protein that promotes transport of cholesterol (Manna and Stocco, 2005; Geslin and Auperin, 2004), and Apolipoprotein B and the Apolipoprotein CI precursor, both components of transported cholesterol particles (Stipanuk, 2000; Babin and Vernier, 1989), were downregulated. The expression of *delta-6 fatty acyl desaturase*, whose protein product catalyzes the synthesis of highly unsaturated fatty acids (Tocher and Ghioni, 1999; Nakamura and Nara, 2003), was downregulated in the liver, and *H-FABP* (heart-type fatty acid binding protein), whose protein product plays a role in fatty acid uptake by the skeletal muscles (Glatz et al., 2003; Ando et al., 1998), was downregulated in the muscle.

4. Discussion

Mammalian studies have demonstrated clear correlations between chronic immune stimulation and muscle wasting (Tisdale, 2002). Humans whose immune systems are chronically stimulated, as in cases of AIDS and other autoimmune diseases, several types of cancer, or tissue injury, often battle muscle wasting more severe than can be explained by the loss of appetite that accompanies their condition (Tisdale, 2002). *In vivo* mouse models of cachexia have uncovered a molecular specificity to muscle wasting in which proinflammatory cytokines, upregulated during immune stimulation, directly inhibit *MyoD* and *myosin*, two genes necessary for muscle growth and maintenance (Acharyya et al., 2004; Guttridge et al., 2000). Since the aquaculture industry focuses significant effort on selection programs for aggressive immune response, a project was undertaken to examine the effects of chronic immune stimulation in rainbow trout.

Expression levels of *TNFα* during this trial indicate that chronic immune stimulation was achieved. For most of the time points, expression was increased in groups receiving LPS injections. We did not detect spikes in expression, which would have indicated repeated acute responses brought on by each LPS injection. Results from this study indicate that chronic immune stimulation reduces growth efficiency in rainbow trout, albeit in a manner somewhat different from that characterized in mammalian systems. Interestingly, the extent of muscle wasting was also not as severe in chronically immune stimulated fish as has been observed in humans or animal models of the syndrome (approximately 5% reduction in fish, compared to approximately 20% in mouse models (Acharyya et al., 2004; Yasumoto et al., 1995) and up to 80% in humans (Tisdale, 2002)). This suggests that fish may be more resistant than mammals to muscle wasting resulting from chronic immune stimulation.

One possible explanation for the apparent resistance may lie in the high content of fish oil ingested by the fish in this study. Eicosapentanoic acid (EPA), a polyunsaturated fatty acid present in fish oil, has been shown to be an attenuator of cancer cachexia in humans (Wigmore et al., 2000), likely because it downregulates the release of proinflammatory cytokines (Endres et al., 1989;

Trebble et al., 2003; Zhao et al., 2004). While specific data on the effect of EPA on *TNFα* expression levels in rainbow trout is lacking, n-3 fatty acid-derived eicosanoids have been shown to lower the immune response in several fish species (Kiron et al., 2004; Rowley et al., 1995; Li et al., 1994). In the current chronic immune stimulation trial, EPA represented 12.5% of the total lipid content of the diet used in this study, well above the 10% defined requirement of EPA and DHA combined that has been reported for rainbow trout (National Research Council, 1993). If fatty acids can attenuate the response to chronic immune stimulation in rainbow trout by downregulating the release of cytokines such as TNFα, then the effect of chronic immune stimulation on muscle may be attenuated as well. The upregulation in *TNFα* expression seen in this study may well have been enhanced if a diet with reduced levels of eicosanoids and other fatty acids were used.

A second explanation may lie in the environment of fish and their response to immune stimuli. In the wild, fish are constantly exposed to endotoxins, likely making them less responsive upon exposure to endotoxins than non-fish animals (Adams et al., 1996; Arinc et al., 2000). While the use of LPS as an immunostimulant in this study did produce increases in *TNFα* mRNA expression, it is possible that initiation of the signaling events that result in muscle loss requires higher levels and a wider range of proinflammatory gene expression than what was induced in this study. The immune response in humans suffering diseases or injury with which cachexia is normally seen are also presumably more complicated. In this study, the immune response was initiated by the injection of one specific endotoxin rather than the global cellular alterations caused by, for example, a cancerous tumor.

Teleost fish display indeterminate muscle growth, which may also partially explain why the extent of muscle wasting in chronically immune-stimulated fish was not as severe as has been observed in humans and animal models. In mammals, there is very little post-natal myoblast proliferation (Parker et al., 2003), however in fish, muscle growth continues by both myoblast proliferation and myotube hypertrophy for the duration of the fish's life (Rowlerson and Veggetti, 2001). Since expression of several genes considered to be positive signals for muscle growth, such as the MRFs and MEFs, continues throughout the lifespan of rainbow trout (Johansen and Overturf, 2005), the signals promoting immune-stimulated growth suppression may be attenuated. In mammals, those same signals may have a more profound consequence in muscle since it is not actively growing.

Since the mechanism of muscle loss in chronically immune stimulated rainbow trout appears to be less myosin-specific than that of mammals, it is likely that a more global nutrient partitioning mechanism ultimately altered these responses. Lending support for this theory is the decreased lipid content in muscles of the chronically immunostimulated fish. A decrease in lipid content is common in humans with cachexia, and is thought to be driven by increased levels of LMF (lipid mobilizing factor) and an induction of lipolysis by circulating cytokines (Tisdale, 1999; Tijerina, 2004). An increased energy demand to maintain the immune response induced by LPS injections could explain the decreases in weight gain and muscle ratio as compared to the pair-fed, non-stimulated fish in this study. However, to date, limited studies have examined the energy demands of chronically immune-stimulated fish or made attempts to identify the underlying mechanisms controlling nutrient partitioning.

Use of the GRASP 16,008-sequence microarray chip to compare gene expression in chronically immune-stimulated fish versus those not stimulated was intended to identify possible mechanisms that could account for the observed muscle loss. Similar studies in mammals have used the same approach to examine muscle wasting and effects of chronic immune stimulation (Lecker et al., 2004; Stevenson et al., 2003). In rat and mouse muscle atrophied from disuse, fasting, or diseases such as cancer cachexia, diabetes mellitus, and uremia, downregulation of genes encoding extracellular matrix proteins, cytoskeletal components, growth factors, muscle structural and contractile proteins, and general transcription and translation factors was observed, while upregulation of genes encoding cytokines, components of cytokine signaling pathways, and protein degradation factors was observed (Lecker et al., 2004; Stevenson et al., 2003). Although equivalent studies in fish are lacking, one study has addressed the effect of LPS on gene expression in cultured rainbow trout macrophage cells. In that study, *in vitro* LPS treatment upregulated genes involved in macrophage induction and extracellular matrix remodeling, while genes involved in cytoskeletal structure, energy metabolism, and translation were downregulated (MacKenzie et al., 2006). Although the cell type tested was different than that tested in our study, it nonetheless demonstrates the effect of LPS on gene expression.

In agreement with MacKenzie et al. (2006), results from the microarray in the current study lend additional molecular support for a role of altered energy metabolism following LPS treatment. Expression of *delta-6 fatty acyl desaturase*, which catalyzes the rate-limiting step in the synthesis of highly unsaturated fatty acids (Tocher and Ghioni, 1999; Nakamura and Nara, 2003), and *H-FABP*, thought to regulate fatty acid uptake in skeletal muscle (Glatz et al., 2003; Ando et al., 1998) were downregulated, suggesting that fatty acid synthesis and uptake are concomitantly downregulated. Also, expression of genes encoding cholesterol transport proteins (Manna and Stocco, 2005; Geslin and Auperin, 2004; Babin and Vernier, 1989), such as two apolipoproteins and StAR, were downregulated, suggesting that cholesterol is not being transported normally. Downregulation of lipid transport and storage genes is consistent with the decreased lipid content of chronically immune-stimulated fish in this study, and further supports the idea of increased energy demand and decreased need for energy storage in chronically immune-stimulated fish. A more extensive analysis of energy and fatty acid metabolism during chronic immune stimulation in fish is necessary to further examine these ideas.

Given the large role that the ubiquitin–proteasome protein degradation pathway is thought to play in immune-stimulated muscle wasting (Lecker et al., 1999), the lack of fold-expression changes in genes associated in this pathway is surprising. One possible explanation for these results is that protein degradation may not be a major factor in the muscle response observed for the chronically immunostimulated rainbow trout in this study. In support of this notion, expression of *calpastatin*, an inhibitor of the calpains, was shown by the array to be upregulated with LPS treatment, suggesting that the calpain protein degradation

pathway is not responsible for the observed muscle response. In fact, in a transgenic mouse overexpressing *calpastatin* in skeletal muscle, a marked resistance to muscle wasting was observed (Tidball and Spencer, 2002). Further analysis confirmed that overexpression of *calpastatin* inhibited the switch from the slow-type myosin heavy chain to the fast-type myosin heavy chain, a process normally observed during muscle atrophy, including that caused by chronic immune stimulation (Tidball and Spencer, 2002; Diffee et al., 2002). This may also explain the relatively minor reduction in muscle ratio in this study compared to some models of cachexia in mice, as well as the lack of detection of a change in myosin expression.

Of additional interest, results obtained from the microarray experiment allude to changes in Ca^{++}-mediated signal transduction during chronic immune stimulation. The expression of the Ca^{++}-regulated molecules encoded by *S100A1-like*, which inhibits the assembly of desmin and glial fibrillary acidic protein (GFAP) into intermediate muscle filaments (Garbuglia et al., 1999), and *Calcyclin Binding Protein*, which binds to S100-family proteins and is a component of a pathway regulating β-catenin degradation (Filipek et al., 2002), was increased, while *IP_3-3KC*, whose protein product metabolizes Ins(1,4,5)P_3 (Pattni and Banting, 2004), and *annexin*, encoding a membrane binding protein which binds to and inhibits S100A1 (Garbuglia et al., 1998), were decreased. Unfortunately, the microarray results are not precise enough to identify additional specific Ca^{++}-regulated pathway components whose expression would further elucidate a mechanism by which this signaling pathway is linked to the observed muscle response. However, taken together with the results of a previous study in this lab that demonstrated increased *calmodulin* expression in the ventricle of enlarged trout hearts (K. A. J. and K. O., unpublished observations), they indicate that more exhaustive analysis of Ca^{++}-mediated muscle growth and degradation pathways is warranted.

Aquaculture is growing more rapidly than all other animal food production sectors (FAO, 2004), and almost one out of every three fish consumed in the world is now farm-raised (Kilpatrick, 2003). Aquaculturists have capitalized upon this growing industry by realizing the importance of efficient production practices, including the institution of selection programs. Results of experiments in this study demonstrate that although the mechanism of immune-stimulated muscle wasting in fish may be somewhat different than that in mammals, selection of strains based on increased levels of immunity may be detrimental to muscle growth. These results may also imply that management practices such as long-term feeding of immunostimulant-containing diets may ultimately reduce production efficiency.

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